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Relaxin requires the angiotensin II type 2 receptor to abrogate renal interstitial fibrosis

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Fibrosis is a hallmark of chronic kidney disease, for which there is currently no effective cure. The hormone relaxin is emerging as an effective antifibrotic therapy; however, its mechanism of action is poorly understood. Recent studies have shown that relaxin disrupts the profibrotic actions of transforming growth factor-β1 (TGF-β1) by its cognate receptor, relaxin family peptide receptor 1 (RXFP1), extracellular signal–regulated kinase phosphorylation, and a neuronal nitric oxide synthase–dependent pathway to abrogate Smad2 phosphorylation. Since angiotensin II also inhibits TGF-β1 activity through its AT2 receptor (AT_2R), we investigated the extent to which relaxin interacts with the AT_2R. The effects of the AT_2R antagonist, PD123319, on relaxin activity were examined in primary rat kidney myofibroblasts, and in kidney tissue from relaxin-treated male wild-type and AT_2R-knockout mice subjected to unilateral ureteric obstruction. Relaxin's antifibrotic actions were significantly blocked by PD123319 *in vitro* and *in vivo*, or when relaxin was administered to AT_2R-knockout mice. While heterodimer complexes were formed between RXFP1 and AT_2Rs independent of ligand binding, relaxin did not directly bind to AT_2Rs but signaled through RXFP1-AT_2R heterodimers to induce its antifibrotic actions. These findings highlight a hitherto unrecognized interaction that may be targeted to control fibrosis progression.

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Characterized by an excessive accumulation of the extracellular matrix (ECM), primarily collagen, fibrosis is a universal response to chronic injury and inflammation in the kidney.[1,2] Prolonged exposure to pathological stimuli and/or profibrotic cytokines causes significant disruption to the regulatory processes that control the rate at which the extracellular matrix is synthesized and degraded, where an imbalance between extracellular matrix synthesis and degradation results in excessive collagen deposition at the site of injury.[3] A failure to resolve this process causes significant nephron destruction leading to progressive organ dysfunction and failure, with damage dependent on the extent of fibrogenesis.[1–3] Angiotensin (Ang) II and transforming growth factor-β1 (TGF-β1) are among the most potent cytokines that drive this pathological process.[4,5]

Despite fibrosis being the final common pathway for all forms of renal disease and an inevitable feature of end-stage kidney failure, there are currently no effective treatments to ameliorate the structural and functional changes that it causes. Furthermore, the cellular and molecular events that underlie this process are poorly understood. Thus, the identification of agents that can alter collagen turnover and remodeling to prevent or even reduce the fibrosis that accompanies progressive renal disease is key to both understanding the cellular and molecular pathways involved and to developing novel treatment strategies.

The ovarian and cardiovascular hormone, relaxin, has emerged as a rapid-acting but safe antifibrotic that ameliorates renal fibrosis in several experimental models, regardless of etiology.[6–9] Although clinical trials have recently explored its vasodilatory benefits in acute heart failure,[10] clinical assessment of the antifibrotic potential of relaxin is less well developed. Despite end-stage kidney disease being progressive, it can take a decade to develop, making it particularly difficult to design, fund, and run trials with hard end points. To this end, a thorough understanding of the signal-transduction mechanisms involved in the antifibrotic

actions of relaxin will significantly facilitate the development of novel therapeutic targets for intervention and design of better clinical trials.

Recent studies have demonstrated that human gene-2 (H2) relaxin (the major stored and circulating form of human relaxin) signals through its cognate G-protein-coupled receptor relaxin family peptide receptor 1 (RXFP1) to activate extracellular signal–regulated kinase phosphorylation (pERK)1/2 and a neuronal nitric oxide (NO) synthase (nNOS)-NO-cyclic guanosine monophosphate (cGMP)–dependent pathway in human[11] and rat[12,13] renal myofibroblasts to inhibit TGF-β1 activity, at the level of Smad2 phosphorylation (pSmad2), an intracellular protein that promotes the profibrotic actions of TGF-β1.[11,14] This in turn inhibits TGF-β1-induced myofibroblast differentiation and myofibroblast-derived aberrant matrix/collagen production,[11,12] while allowing for an upregulation of the matrix metalloproteinases (MMP-1/MMP-13, MMP-2, and MMP-9) that are associated with the breakdown of existing collagen.[13] Furthermore, by suppressing the TGF-β1/pSmad2 axis that inhibits iNOS activity in myofibroblasts,[15] H2 relaxin is able to release iNOS, which through higher levels of NO specifically contributes to the MMP-promoting actions of the hormone.[13]

To further understand how H2 relaxin inhibits the profibrotic influence of TGF-β1 in various fibroblast culture models,[11–13,16–19] this study sought to find the points at which H2 relaxin interacts with the well-established Ang II-TGF-β1 system.[20] Ang II is a well-known vasoconstrictor that increases blood pressure, and a potent profibrogenic cytokine.[21] These classical actions of Ang II along with its ability to promote TGF-β1 activity are mediated through the angiotensin type 1 receptor (AT_1R). Conversely, Ang II also negatively regulates TGF-β1 activity and tissue remodeling by acting at the angiotensin type 2 receptor (AT_2R).[22,23] Both AT_1Rs and AT_2Rs are expressed in the kidney. Given our previous findings that H2 relaxin separately inhibits the collagen-stimulatory actions of Ang II or TGF-β1 in other organs,[19] we aimed to examine the interaction between H2 relaxin and the AT_2R to determine how this influences the profibrotic actions of TGF-β1. The experiments were performed in primary renal myofibroblasts *in vitro* and in an experimental model of tubulointerstitial renal fibrosis *in vivo*.

RESULTS

The antifibrotic actions of H2 relaxin are blocked by the AT_2R antagonist PD123319 *in vitro*

Consistent with our previous findings,[12,13] treatment of renal myofibroblasts with recombinant H2 relaxin (100 ng/ml; 16.8 nmol/l) for 72 h promoted ERK1/2 (p42/p44 mitogen-activated protein kinase (MAP kinase)) phosphorylation (pERK1/2), nNOS expression, and nNOS phosphorylation (pnNOS) by 0.8- to 1-fold (Figure 1a) and levels of collagen-degrading MMPs (MMP-9, MMP-2, MMP-13) by 0.85- to 1.3-fold (Figure 1c), while inhibiting TGF-β1 expression, pSmad2, and α-smooth muscle actin (α-SMA) levels (a marker of myofibroblast differentiation) by 0.5-fold (Figure 1b; Supplementary Figure S1 online) (all $P<0.01$ vs. respective values from untreated cells). All these H2 relaxin–induced effects were blocked by the AT_2R antagonist PD123319 (0.1 μmol/l; all $P<0.01$ vs. H2 relaxin treatment), whereas PD123319 (0.1 μmol/l) alone did not affect basal levels of the various parameters measured (Figure 1).

The antifibrotic actions of H2 relaxin are abrogated by the absence of, or blockade of, AT_2R *in vivo*

To substantiate the above findings in isolated cells (Figure 1), *in vivo* studies examined male $AT_2R^{+/+}$ (wild-type) and $AT_2R^{-/y}$ (knockout) mice after unilateral ureteric obstruction (UUO). Their kidneys were assessed at day 2 (when fibrogenesis can be measured) and day 5 after injury (when renal fibrosis is well established). Mice were also pretreated or delayed-treated with H2 relaxin (0.5 mg/kg per day) alone or in combination with PD123319 (3 mg/kg per day). Total kidney collagen concentration (Figure 2a), collagen IV staining (Figure 2b), and collagen I staining (Figure 2c) were all progressively increased in both $AT_2R^{+/+}$ and $AT_2R^{-/y}$ mice after UUO, but were 0.15- to 1.1-fold greater in $AT_2R^{-/y}$ mice compared with UUO–injured $AT_2R^{+/+}$ mice by day 5 after injury (all $P<0.05$ vs. respective measurements from injured $AT_2R^{+/+}$ mice). Immunohistochemically stained sections of $AT_2R^{-/y}$ mice showed an expanded interstitium, with increased deposition of collagen IV (Figure 2b) and collagen I (Figure 2c) by day 5 after injury. Pretreatment and delayed treatment of $AT_2R^{+/+}$ mice with recombinant H2 relaxin significantly reduced both renal collagen concentration (Figure 2a) and collagen IV staining (Figure 2b) by 0.33- to 0.4-fold, and further reduced collagen I staining (Figure 2c) to levels beyond those measured in day 5 (and even day 2) post-UUO animals (all $P<0.05$ vs. respective measurements from day 5 UUO $AT_2R^{+/+}$ mice). These collagen-inhibitory effects of H2 relaxin treatment were completely lost when it was administered to $AT_2R^{-/y}$ mice, or when it was coadministered with PD123319 to $AT_2R^{+/+}$ mice (all $P<0.01$ vs. respective values from H2 relaxin–pretreated and delayed-treated $AT_2R^{+/+}$ mice; Figure 2).

Kidney protein extracts from day 5 UUO controls and mice that were pretreated with H2 relaxin ± PD123319 were further evaluated for additional targets of relaxin activity. Compared with $AT_2R^{+/+}$ mice, the kidneys of day 5 UUO–injured $AT_2R^{-/y}$ mice had 0.5- to 0.6-fold lower pERK1/2, nNOS, pnNOS (Figure 3a), and MMP-13 levels (Figure 3b), but 1- to 1.3-fold increased TGF-β1, pSmad2, and α-SMA (Figure 3b). Pretreatment of $AT_2R^{+/+}$ mice with recombinant H2 relaxin caused a significant elevation in renal pERK and pnNOS (by 1- to 1.7-fold; Figure 3a) and MMP-13 levels (by 1.6-fold; Figure 3b), but reduced TGF-β1 and α-SMA expression, as well as pSmad2 phosphorylation (by 0.5- to 0.6-fold of that in day 5–injured $AT_2R^{+/+}$ mice; Figure 3b; all $P<0.05$ vs. respective values from day 5–injured $AT_2R^{+/+}$ mice). Again, the effects of relaxin were abrogated when it was administered to $AT_2R^{-/y}$ mice or when it was

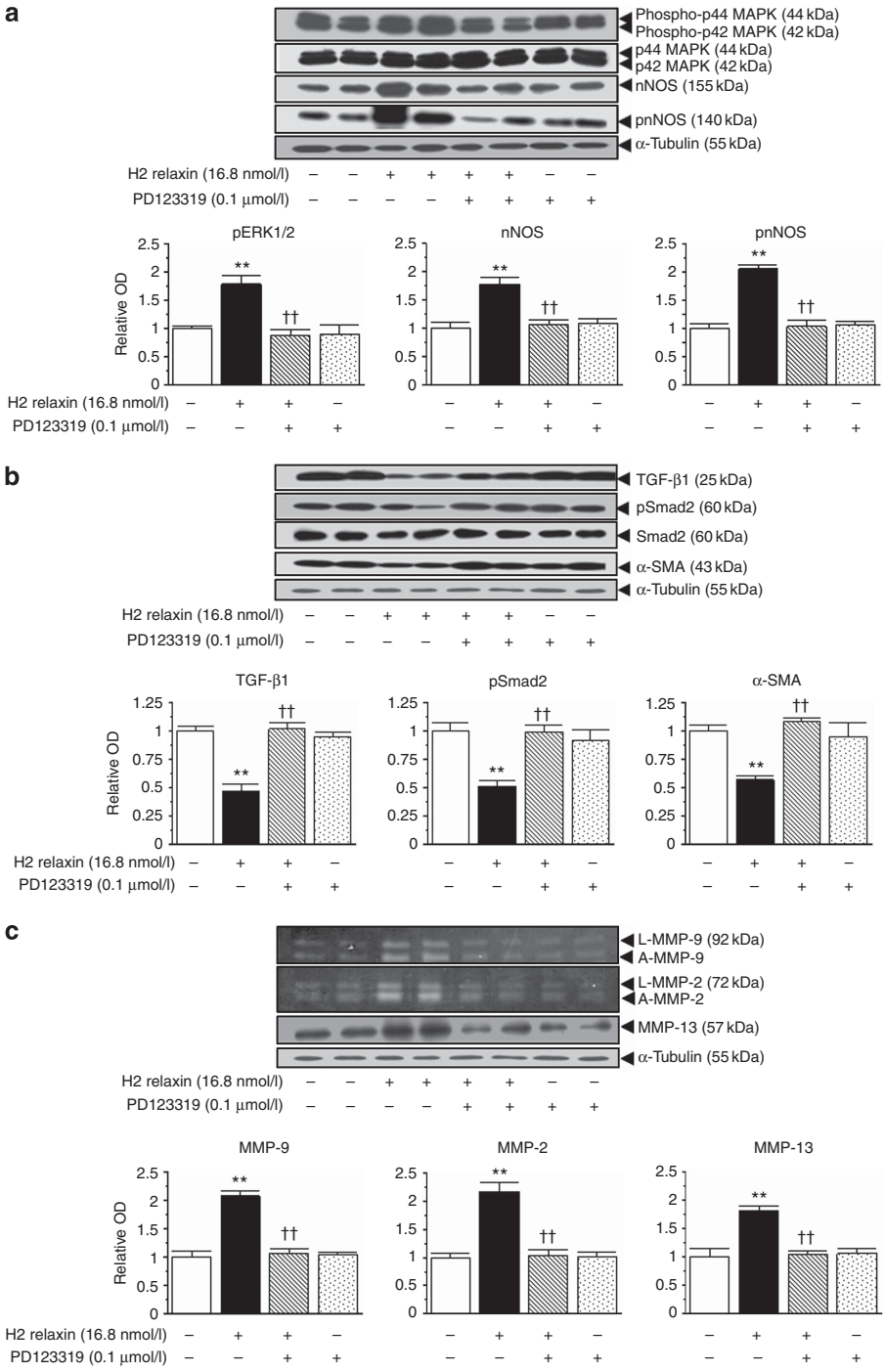

Figure 1 | The antifibrotic actions of human gene-2 (H2) relaxin are blocked by the AT2 receptor (AT_2R) antagonist PD123319 *in vitro*. Representative western blots of renal (**a**) phosphorylated (phospho-) p44 and p42 mitogen-activated protein kinase (MAPK) (extracellular signal–regulated kinase 1/2 phosphorylation (pERK1/2)), total p44 and p42 MAPK (ERK1/2), unphosphorylated neuronal nitric oxide synthase (nNOS), nNOS phosphorylation (pnNOS), and α-tubulin; (**b**) transforming growth factor-β1 (TGF-β1), Smad2 phosphorylation (pSmad2), unphosphorylated Smad2, α-smooth muscle actin (SMA), and α-tubulin; and (**c**) representative zymographs of latent (L) and active (A) matrix metalloproteinase-9 and -2 (MMP-9 and -2) levels and representative western blots of MMP-13 and α-tubulin expression from untreated (control) rat renal myofibroblasts and cells treated with H2 relaxin (16.8 nmol/l) alone, H2 relaxin (16.8 nmol/l), PD123319 (0.1 μmol/l), or PD123319 (0.1 μmol/l) alone after 72 h in culture. The total (**a**) p44 and p42 MAPK (ERK1/2), (**b**) unphosphorylated Smad2, and (**a–c**) α-tubulin blots were included to demonstrate the quality and equivalent loading of protein samples. Also shown is the relative mean ± s.e.m. Optical density (OD) levels of (**a**) pERK1/2 (corrected for total ERK1/2 levels), nNOS, and pnNOS (both corrected for α-tubulin levels); (**b**) TGF-β1, α-SMA (both corrected for α-tubulin levels), and pSmad2 (corrected for Smad2 levels); and (**c**) MMP-9, MMP-2, and MMP-13 (corrected for α-tubulin levels) from each of the groups studied, as determined by densitometry scanning (from $n = 4$ experiments conducted in duplicate), to that of the untreated group, which was expressed as 1 in each case. $^{**}P < 0.01$ vs. untreated cells; $^{\dagger\dagger}P < 0.01$ vs. H2 relaxin alone-treated cells.

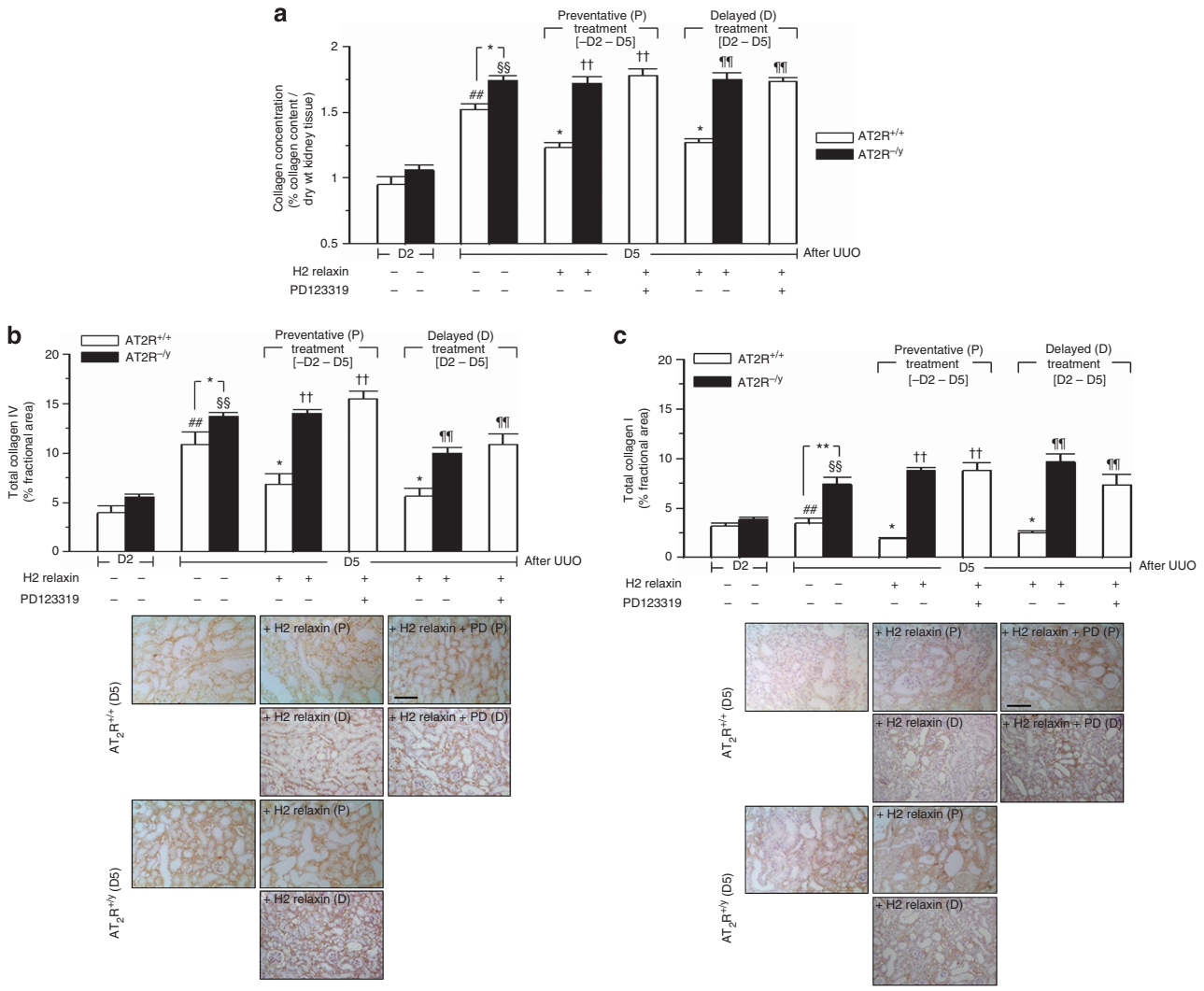

Figure 2 | The collagen-inhibitory actions of human gene-2 (H2) relaxin were abrogated by the absence of, or blockade of, AT_2 receptors (AT_2Rs) *in vivo*. Shown is the relative mean ± s.e.m. (**a**) renal collagen concentration (% collagen content/dry weight kidney tissue), (**b**) collagen IV staining (as a % of the fractional area), and (**c**) collagen I staining (as a % of the fractional area) from day 2 (D2)– and day 5 (D5)–injured $AT_2R^{+/+}$ and $AT_2R^{-/y}$ mice; D5-injured $AT_2R^{+/+}$ and $AT_2R^{-/y}$ animals that were pretreated (from 2 days before (−D2) to injury) or delayed-treated (from D2 after unilateral ureteric obstruction (UUO)) with H2 relaxin (0.5 mg/kg per day); and D5-injured $AT_2R^{+/+}$ mice that were pretreated or delayed-treated with H2 relaxin (0.5 mg/kg per day) and PD123319 (3 mg/kg per day), from $n = 5$–7 mice per treatment group. As a reference, uninjured $AT_2R^{+/+}$ mice have renal collagen concentrations of 0.75–0.85%. Also shown are representative images of (**b**) collagen IV and (**c**) collagen I staining from the preventative (P) and delayed (D) treatment groups investigated at D5 after injury. Bar = 100 μm. $^{\#\#}P < 0.01$ vs. D2-injured $AT_2R^{+/+}$ mice; $^{\S\S}P < 0.01$ vs. D2-injured $AT_2R^{-/y}$ mice; $^{*}P < 0.05$ vs. D5-injured $AT_2R^{+/+}$ mice; $^{\dagger\dagger}P < 0.01$ vs. D5-injured $AT_2R^{+/+}$ mice pretreated with H2 relaxin alone; $^{\P\P}P < 0.01$ vs. D5-injured $AT_2R^{+/+}$ mice delayed-treated with H2 relaxin alone.

coadministered with PD123319 to $AT_2R^{+/+}$ mice (all $P < 0.01$ vs. respective values from H2 relaxin–treated $AT_2R^{+/+}$ mice; Figure 3), confirming that the AT_2R was necessary for H2 relaxin to mediate its antifibrotic actions.

Constitutive heterodimers are formed between RXFP1 and the AT_2R

Bioluminescence resonance energy transfer (BRET) saturation assays carried out in human embryonic kidney (HEK)293 cells to examine the formation of dimers between RXFP1 and AT_2R indicated that constitutive dimerization occurred between RXFP1 and AT_2R. Saturation curves were constructed by keeping a constant amount of Rluc8-tagged AT2R and increasing the amount of Venus-tagged RXFP1. The BRET curve detecting RXFP1 and AT2R interactions showed saturation with a maximum BRET ratio of ~0.1 (Figure 4a), providing strong evidence that RXFP1 and AT_2Rs formed heterodimers. In contrast, no dimerization was observed between RXFP1 and the thyrotropin-releasing hormone receptor 1, which was used as a negative control (Figure 4a).

The effect of recombinant H2 relaxin and Ang II on the RXFP1-AT_2R dimers was then examined using real-time kinetic BRET assays. The addition of H2 relaxin (30 nmol/l), Ang II (1 μmol/l), or a combination of H2 relaxin and Ang II

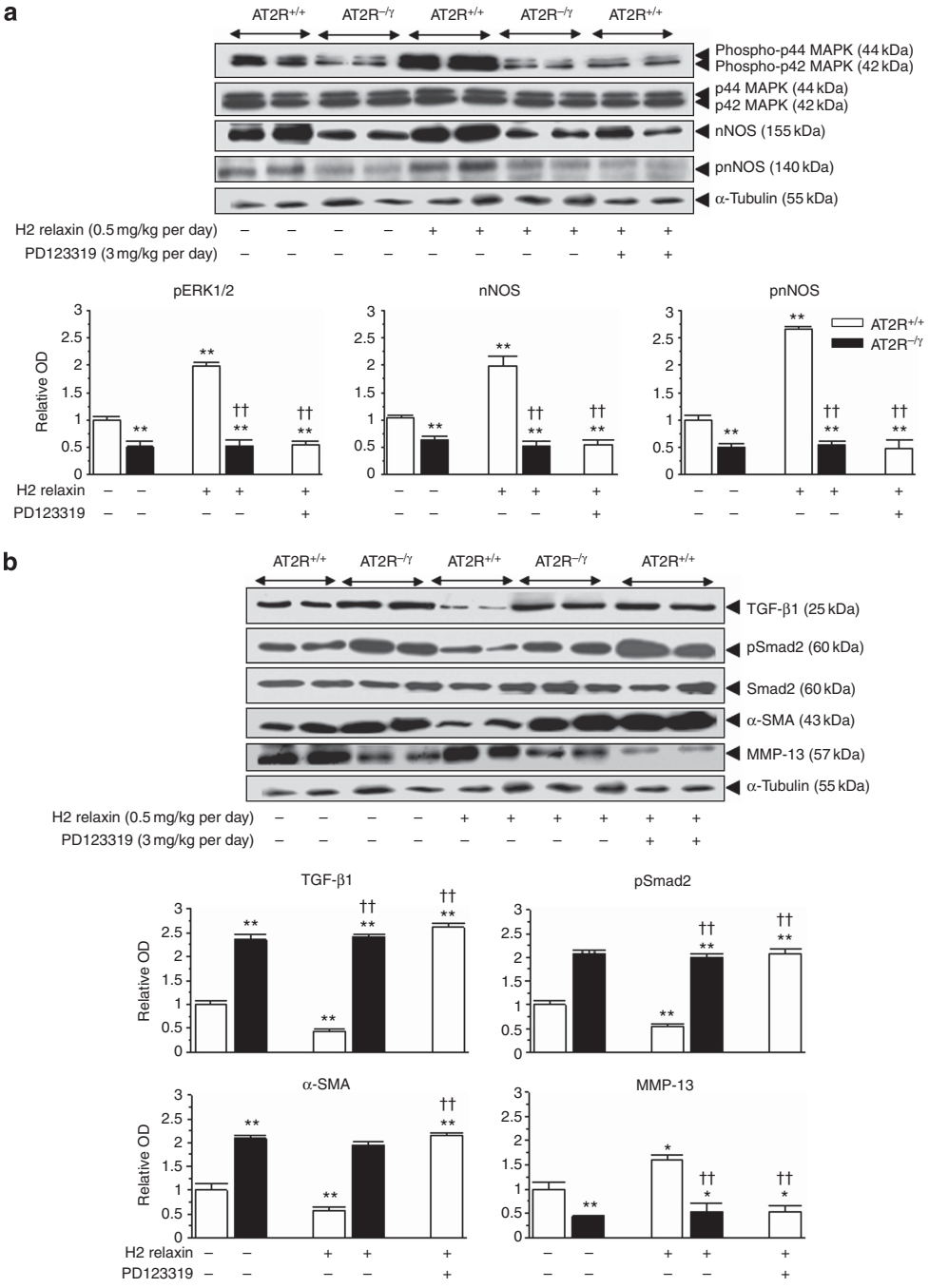

Figure 3 | The antifibrotic actions of human gene-2 (H2) relaxin are abrogated by the absence of, or blockade of, AT_2 receptors (AT_2Rs) *in vivo*. Representative western blots of renal (**a**) phosphorylated (phospho-) p44 and p42 mitogen-activated protein kinase (MAPK) (ERK1/2 phosphorylation (pERK1/2)), total p44 and p42 MAPK (ERK1/2), unphosphorylated neuronal nitric oxide synthase (nNOS), nNOS phosphorylation (pnNOS), and α-tubulin; (**b**) transforming growth factor-β1 (TGF-β1), Smad2 phosphorylation (pSmad2), unphosphorylated Smad2, α-smooth muscle actin (α-SMA), matrix metalloproteinase-13 (MMP-13), and α-tubulin, from day 5–injured $AT_2R^{+/+}$ and $AT_2R^{-/y}$ mice ± pre-treatment with H2 relaxin (0.5 mg/kg per day) alone; and day 5–injured $AT_2R^{+/+}$ mice pre-treated with H2 relaxin (0.5 mg/kg per day) and PD123319 (3 mg/kg per day). The total (**a**) p44 and p42 MAPK (ERK1/2), (**b**) unphosphorylated Smad2, and (**a** and **b**) α-tubulin blots were included to demonstrate the quality and equivalent loading of protein samples. Also shown is the relative mean ± s.e.m. optical density (OD) levels of (**a**) pERK1/2 (corrected for total ERK1/2 levels), nNOS, and pnNOS (both corrected for α-tubulin levels); (**b**) TGF-β1, α-SMA, MMP-13 (all corrected for α-tubulin levels), and pSmad2 (corrected for Smad2 levels) from each of the groups studied, as determined by densitometry scanning (from $n = 5$–6 mice/treatment group), to that of the day 5–injured $AT_2R^{+/+}$ mouse group, which was expressed as 1 in each case. $^{**}P < 0.01$ vs. day 5–injured $AT_2R^{+/+}$ mice; $^{\dagger\dagger}P < 0.01$ vs. day 5–injured $AT_2R^{+/+}$ mice pretreated with H2 relaxin alone.

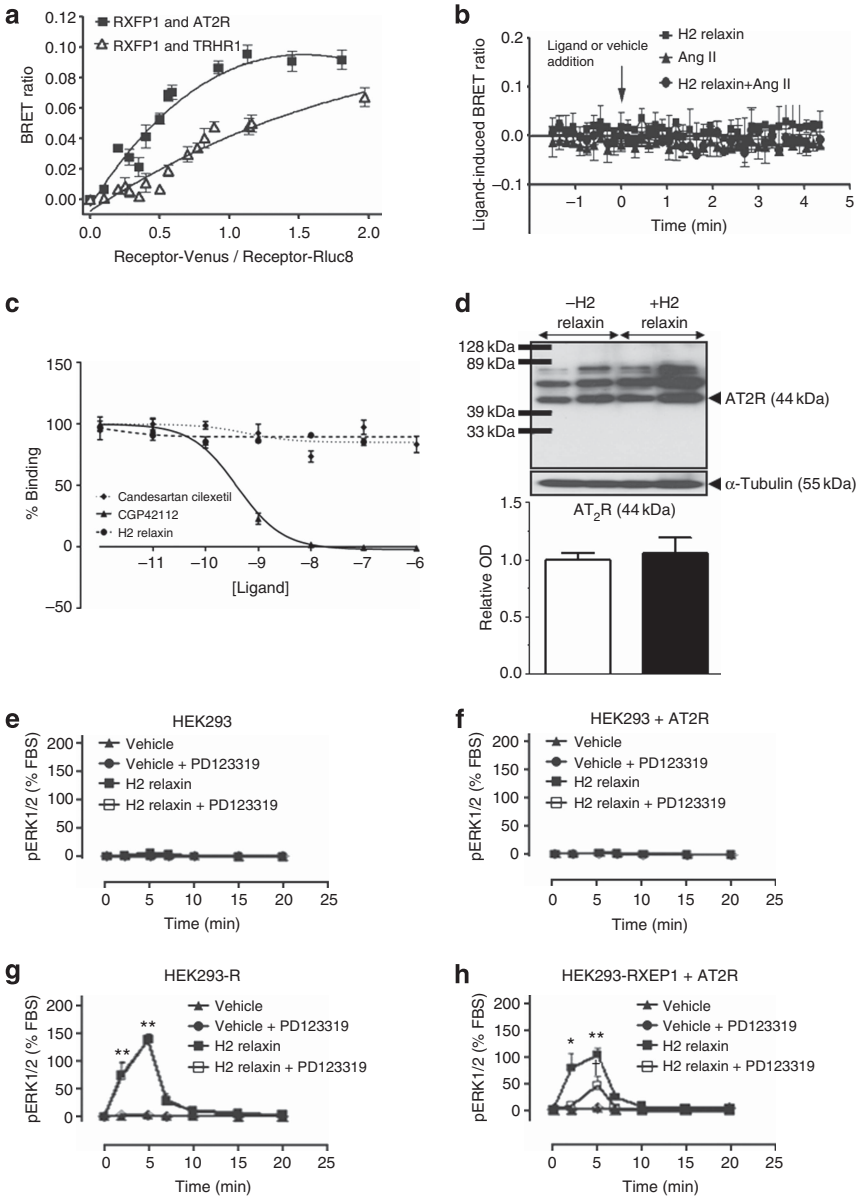

Figure 4 | Human gene-2 (H2) relaxin does not directly interact with the AT_2 receptor (AT_2R). (**a**) Bioluminescence resonance energy transfer (BRET) saturation curves and (**b**) real-time kinetic BRET curves detecting (**a**) ligand-independent and (**b**) ligand-induced interactions between relaxin family peptide receptor 1 (RXFP1) and AT_2Rs. Human embryonic kidney (HEK)293 cells were transiently co-transfected with (**a** and **b**) RXFP1-Venus and AT2R-Rluc8 or (**a**) RXFP1-Venus and thyrotropin-releasing hormone receptor 1 (TRHR1)-Rluc8 (negative control). The BRET ratios are plotted as a function of the expression ratio of (Receptor-Venus)/(Receptor-Rluc8). (**b**) The ligand-induced BRET ratios were detected before and after treatment with agonist (H2 relaxin (30 nmol/l); Ang II (1 μmol/l); H2 relaxin (30 nmol/l) plus angiotensin II (Ang II) (1 μmol/l) or vehicle (phenol red-free + 10% fetal bovine serum (FBS) + 0.01% bovine serum albumin) and calculated as described in Materials and Methods. Data shown (**a** and **b**) are the mean ± s.e.m. of $n = 3$ experiments performed in triplicate. Competition binding curves for CGP42112, candesartan cilexetil, and H2 relaxin (**c**) were also generated from HEK293 cells stably transfected with AT_2Rs. Data shown (**c**) are the mean ± s.e.m. of $n = 4$ performed in triplicate. Additionally shown are representative western blots of renal AT_2R expression (**d**) from untreated versus H2 relaxin (16.8 nmol/l)–treated rat renal myofibroblasts (from $n = 4$ experiments in duplicate). Functional responses of RXFP1-AT_2R dimers in response to H2 relaxin, using the AlphaScreen extracellular signal–regulated kinase 1/2 (ERK1/2) phosphorylation assay—shown are the effects of (**e**) vehicle (20 mmol/l sodium acetate buffer, pH 5.0) alone, vehicle + PD123319 (1 μmol/l), H2 relaxin (30 μmol/l) alone, and H2 relaxin (30 μmol/l) + PD123319 (1 μmol/l) in untransfected HEK293 cells, (**f**) HEK293 cells transfected with AT_2Rs, (**g**) HEK293 cells expressing RXFP1 (HEK-RXFP1), and (**h**) HEK-RXFP1 cells transfected with AT_2Rs on ERK1/2 phosphorylation (pERK1/2) (p42/p44 mitogen-activated protein kinase) activity over a 60-min period (of which responses over the first 20 min are shown). All data (**e**–**h**) are expressed as the mean ± s.e.m. of $n = 4$–6 experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. respective measurements from vehicle-treated cells; $^{\dagger}P < 0.05$ vs. respective measurement from H2 relaxin–treated cells. OD, optical density.

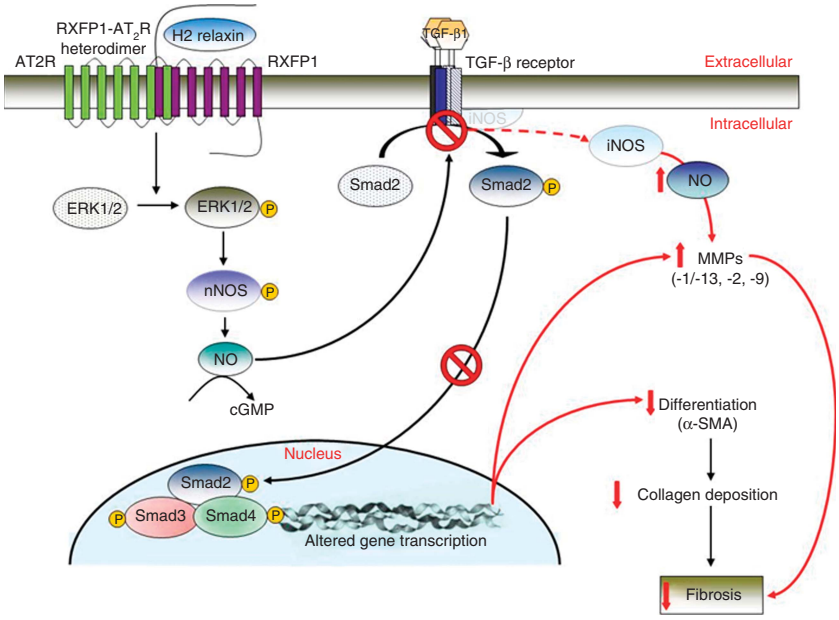

Figure 5 | A schematic illustration of the proposed signal-transduction mechanisms of human gene-2 (H2) relaxin's antifibrotic actions, via relaxin family peptide receptor 1-AT2 receptor (RXFP1-AT_2R) heterodimers. Previous findings from primary renal myofibroblasts[11–13] demonstrated that H2 relaxin signaled through a extracellular signal–regulated kinase 1/2 phosphorylation (pERK1/2) and a neuronal nitric oxide (NO) synthase (nNOS)-NO-cyclic guanosine monophosphate (cGMP)–dependent pathway to inhibit Smad2 phosphorylation (pSmad2) and translocation to the nucleus as a means of disrupting transforming growth factor-β1 (TGF-β1) activity. This in turn ameliorated TGF-β1-mediated myofibroblast differentiation and myofibroblast-induced aberrant collagen deposition (the basis of fibrosis), while allowing for an upregulation of matrix metalloproteinases (MMPs) associated with collagen degradation. Furthermore, the H2 relaxin–induced suppression of the TGF-β1, which itself inhibits inducible NOS (iNOS) expression in myofibroblasts, releases iNOS, which through higher levels of NO specifically contributes to the MMP–promoting actions of the antifibrotic hormone. Our findings from the current study now suggest that H2 relaxin signals through constitutive RXFP1-AT_2R heterodimers and a RXFP1/AT_2R-pERK1/2-nNOS-NO-cGMP pathway to inhibit the TGF-β1/pSmad2 axis and the downstream effects of TGF-β1, allowing it to induce the various actions detailed above (as indicated by the red arrows). α-SMA, α-smooth muscle actin.

had no effect on existing heterodimers between RXFP1 and the AT_2R (Figure 4b).

H2 relaxin does not directly interact with the AT_2R

To determine whether H2 relaxin displayed any direct binding affinity for the AT_2R, competition-binding assays were carried out with H2 relaxin, an AT_2R agonist CGP42112, and an AT_1R antagonist candesartan in HEK293 cells expressing the AT_2R (Figure 4c). Only CGP42112, but not H2 relaxin or candesartan, was able to bind to the AT_2R (Figure 4c), confirming that H2 relaxin did not directly bind to the AT_2R. Furthermore, western blotting of AT_2Rs endogenously expressed in primary rat renal myofibroblasts confirmed that H2 relaxin (16.8 nmol/l; 72 h) did not alter AT_2R receptor expression (Figure 4d), despite the antibody used detecting the expected 44-kDa product and two larger-molecular-weight products.

H2 relaxin mediates the cross-talk through RXFP1-AT_2R dimerization at the level of pERK1/2

To determine the downstream consequences of RXFP1-AT_2R dimerization (Figure 4a) and whether this was possibly linked to the ability of H2 relaxin to stimulate pERK1/2 signaling downstream of RXFP1 (Figure 1),[12,13] untransfected HEK293 cells or HEK293-expressing RXFP1 (HEK-RXFP1) and transfected with AT_2R were measured for pERK1/2 activity using the AlphaScreen ERK1/2 phosphorylation assay.[24] H2 relaxin (30 nmol/l, a dose previously used to stimulate intracellular signaling[12,25]) did not stimulate any pERK1/2 activity over 60 min, when administered to untransfected cells (Figure 4e) or cells transiently transfected with AT_2Rs (Figure 4f), confirming the findings from the competition-binding assays (Figure 4c) that H2 relaxin did not directly bind to the AT_2R. In contrast, pERK1/2 activity was significantly increased over 2–5 min, when H2 relaxin was administered to HEK-RXFP1 alone (Figure 4g) or to HEK-RXFP1 cells transfected with the AT_2R (Figure 4h) ($P<0.05$ vs. respective vehicle treatment of cells at both time points), before returning to baseline within 10 min. The H2 relaxin–induced stimulation of pERK1/2 in HEK-RXFP1 cells was unaffected by PD123319 (1 μmol/l) (Figure 4g), but was significantly, although not totally, abrogated when the AT_2R was coexpressed (Figure 4h), the latter being consistent with transient AT_2R expression resulting in coexpression (of both receptors) in only a proportion of RXFP1-expressing cells.

DISCUSSION

This study explored the interaction between H2 relaxin-RXFP1[12] and AT_2R[22,23,26–29] activation, which inhibits

TGF-β1 expression and/or activity while exerting cardio/renoprotection through an nNOS-NO-cGMP–dependent pathway. Consistent with our previous findings, we found that H2 relaxin signals through an RXFP1-pERK1/2-nNOS-NO-cGMP–dependent pathway to abrogate the TGF-β1/pSmad2 axis[12,13] and inhibit the profibrotic influence of TGF-β1 on renal myofibroblast differentiation,[12] while regulating collagen-degrading MMPs (MMP-1/-13, MMP-2 and MMP-9).[13] Surprisingly, however, this was completely abolished by the AT_2R antagonist PD123319 at all levels. Furthermore, the antifibrotic actions of relaxin *in vivo*, regardless of whether it was administered before or after UUO, were completely lost when the AT_2R was either absent (in $AT_2R^{-/y}$ mice) or antagonized (with PD123319), confirming that the AT_2R was essential for the antifibrotic actions of H2 relaxin. However, H2 relaxin did not produce these effects by a direct action at the AT_2R, as the peptide did not bind to AT_2Rs or alter the dynamics of already formed RXFP1-AT_2R complexes. Instead, we demonstrated for the first time that H2 relaxin signals through constitutive RXFP1-AT_2R heterodimers to induce its downstream effects,[11–13] resulting in the inhibition of the TGF-β1/pSmad2 axis and hence reduced TGF-β1-induced collagen deposition (the basis of fibrosis) (Figure 5).

These findings were quite striking and may help explain why H2 relaxin only displays its antifibrotic effects under pathological conditions, without affecting normal extracellular matrix and fibroblast function under physiological conditions.[6–9,16–19] As AT_2Rs are expressed at low levels in tissues[22,23,27] and fibroblasts under physiologically quiescent states, but are markedly increased in number and activity under pathological conditions, the increased availability of AT_2Rs in injured/diseased tissues improves the functional importance of these receptors,[22,23,26–29] not only on their own but also through interactions with other receptors. Hence, pathological conditions would be more conducive to RXFP1-AT_2R heteromerization taking place and hence for H2 relaxin to mediate its antifibrotic actions through these RXFP1-AT_2R heteromers. Furthermore, as activation of AT_2Rs decreases AT_1R expression[30] and antagonizes the effects of AT_1R activation,[30,31] our findings suggest that H2 relaxin may also indirectly affect the Ang II–AT_1R–TGF-β1 interaction via effects on RXFP1-AT_2R dimers.

Consistent with our findings here, H2 relaxin reduces Ang II infusion–induced renal oxidative stress, glomerular sclerosis, arterial pressure, and albuminuria in hypertensive rats.[32] These protective effects, however, were lost in animals cotreated with the general NOS inhibitor L-NAME. Along with previous studies demonstrating that nNOS is widely distributed in the kidney and shown to be a marker of renal injury,[33] our current observations suggest that the nNOS-NO pathway is absolutely required for the protective/antifibrotic actions of H2 relaxin in kidney/renal myofibroblasts, and are likely mediated via RXFP1-AT_2R heterodimers.

The accelerated tubulointerstitial fibrosis (represented by increased collagen IV, collagen I, and total collagen concentration) in $AT_2R^{-/y}$ mice subjected to UUO that was measured in this study is consistent with previous findings,[34] confirming that AT_2Rs protect from renal fibrosis. These findings are also consistent with our previous observations that $RXFP1^{-/y}$ mice similarly underwent more rapidly progressive interstitial renal fibrosis after UUO.[12] We have now, however, provided further insights into the mechanisms by which AT_2Rs protected against renal fibrosis progression at the *in vitro* and *in vivo* level, which involved an upregulation of ERK1/2 phosphorylation, nNOS activity (nNOS-mediated NO production), and collagen-degrading MMP levels, and downregulation of TGF-β1/pSmad2, myofibroblast differentiation, and aberrant collagen levels. Taken together, these combined findings suggested that H2 relaxin uses the antifibrogenic and antiremodeling effects of AT_2Rs (via RXFP1-AT_2R dimers) to mediate its antifibrotic actions.

Although our previous studies[12] confirmed that RXFP1 in renal myofibroblasts was essential for H2 relaxin's inhibition of myofibroblast differentiation and myofibroblast-mediated collagen deposition, we now demonstrate that constitutive RXFP1-AT_2R heteromer complexes are formed independently of any ligand (H2 relaxin or Ang II) binding. These findings resemble other heteromerization paradigms among G-protein-coupled receptors.[35,36] In addition, we now demonstrate that H2 relaxin appeared to signal through these constitutive RXFP1-AT_2R heterodimers to induce downstream functional effects, at the level of pERK1/2 (which had previously been shown to be activated by relaxin-RXFP1[12,13] or Ang II-AT_2R[28] to mediate organ protection), to inhibit the TGF-β1/Smad2 axis. Our added finding that pERK1/2 responses were almost completely blocked by PD123319 under conditions where RXFP1-AT_2R dimers are present also suggested that these RXFP1-AT_2R heteromers may be regulated by AT_2R blockade, as a novel means by which the actions of H2 relaxin may be inhibited.

In conclusion, we have demonstrated a novel mechanism by which H2 relaxin disrupts the profibrotic effects of TGF-β1 in activated myofibroblasts. The AT_2R is critically required for H2 relaxin to disrupt the TGF-β1/pSmad2 axis via a RXFP1/AT2R-pERK1/2-nNOS-NO-cGMP–dependent pathway, which in turn inhibits the eventual downstream effects of TGF-β1 on myofibroblast differentiation, aberrant collagen deposition, and collagen-degrading MMP levels. This essential requirement is via the formation of AT_2R-RXFP1 constitutive heterodimers. The relative absence of the AT_2R under normal physiological conditions and its upregulation in models of fibrosis may explain why the antifibrotic effects of H2 relaxin are only seen under pathological conditions. These findings further demonstrate that H2 relaxin acts at multiple levels to disrupt both TGF-β1 signal transduction and the profibrotic interaction between Ang II and TGF-β1. Furthermore, we have identified the AT_2R as a novel therapeutic target that may enhance the antifibrotic potential of H2 relaxin to reduce fibrosis progression.

MATERIALS AND METHODS

Materials

Recombinant H2 relaxin was generously provided by Corthera (San Mateo, CA; a subsidiary of Novartis International AG, Basel, Switzerland) and is bioactive in rats[12,13,18,19] and mice.[12,17,19,37] CGP42112 (AT_2R agonist) was obtained from GL Biochem (Shanghai) (Shanghai, China), whereas candesartan cilexetil (AT_1R antagonist) was obtained from AstraZeneca (Södertälje, Sweden).

Animals

A rat and mouse model of UUO, which mimics the pathology of human progressive renal disease,[38] was used as an experimental model of primary tubulointerstitial fibrosis. In each case, a single ureter was ligated under general anesthesia with the contralateral kidney left intact. Tissue was collected from the obstructed kidneys of male Sprague–Dawley rats (obtained from the Animal Resource Centre, Perth, WA, Australia) for the propagation of renal fibroblasts. Similarly, the role of the renal AT_2R was examined in male littermate $AT_2R^{+/+}$ and $AT_2R^{-/y}$ mice (on an FVB/N background; kindly provided by Professor Lutz Hein, University of Freiburg, Freiburg, Germany; and validated previously[39]), subjected to UUO.

Animals were housed in a controlled environment and maintained on a fixed lighting schedule with free access to rodent lab chow (Barastock Stockfeeds, Pakenham, VIC, Australia) and water. These experiments were approved by the Florey Institute of Neuroscience and Mental Health's and Monash University's Animal Ethics Committees, which adhere to the Australian code of practice for the care and use of laboratory animals for scientific purposes.

Cell culture

Myofibroblasts propagated from fibrotic kidneys[40] of Sprague–Dawley rats, 3 days after UUO were used for these studies, as described before,[12,13,41] as they behave like their counterparts from fibrotic human kidneys, and respond to H2 relaxin in a similar manner as TGF-β1-stimulated human renal fibroblasts.[11] Cells were characterized by immunocytochemical staining. As 100% of cells stained positive for the mesenchymal marker vimentin, and 60–70% of cells positively stained for α-SMA, it was concluded that fibroblasts constituted 100% of the cell population used for experimentation, of which 60–70% were myofibroblasts.

Cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2.2% HEPES, 1% L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml) (Dulbecco's modified Eagle's medium-FBS) at 37°C. All described experiments were independently replicated at least 3–6 separate times in duplicate, with rat renal myofibroblasts used between passages 13 and 18 (during which time they expressed RXFP1 and the AT_2R).

Evaluating the effects of AT_2R blockade on the antifibrotic effects of H2 relaxin *in vitro*

To elucidate whether there was any interaction between H2 relaxin and the AT_2R, rat myofibroblasts were seeded into 12-well plates at an equal density of $1–1.25\times10^5$ cells per well and continuously treated with recombinant H2 relaxin (100 ng/ml; 16.8 nmol/l)[12,13] in the absence or presence of the specific AT_2R antagonist PD123319 (0.1 μmol/l) over 72 h. Untreated cells and cells treated with PD123319 alone (at 0.1 μmol/l) for 72 h were used as appropriate controls. After 72 h, proteins were extracted from cell layers with Trizol reagent (Invitrogen, Carlsbad, CA; according to the manufacturer's instructions) for further analysis.

Evaluating the effects of AT_2R blockade on the antifibrotic effects of H2 relaxin *in vivo*

The importance of the AT_2R in mediating H2 relaxin's antifibrotic actions was further investigated in 6- to 8-week-old male $AT_2R^{+/+}$ and $AT2R^{-/y}$ mice on an FVB/N background, subjected to the UUO–induced model of tubulointerstitial renal fibrosis.[12,37] Subgroups of $AT_2R^{+/+}$ and $AT_2R^{-/y}$ mice ($n=5–6$ per genotype and treatment group) that were subjected to UUO were either left untreated until 2 or 5 days postoperatively (injury controls) or were either pretreated with recombinant H2 relaxin (0.5 mg/kg per day; via subcutaneously implanted osmotic minipumps; model 1007D; Alzet, Cupertino, CA) from 2 days before UUO until 5 days after injury (preventative treatment) or treated with the same dose of H2 relaxin (via 1003D pumps; Alzet) from days 2 to 5 after UUO (delayed treatment). This dose of H2 relaxin had previously been used to successfully prevent/reverse fibrosis progression in various models of renal disease, regardless of etiology,[6–9] and was found to produce ~20 ng/ml of circulating relaxin after 5 days of administration.[42] Further subgroups of $AT_2R^{+/+}$ mice were either pretreated or delayed-treated with H2 relaxin (as above) in combination with PD123319 (3 mg/kg per day;[43] via osmotic minipumps over the 7- or 3-day treatment periods, respectively).

At 2 and 5 days after UUO, all animals were killed by an overdose of anesthetic, and their obstructed kidneys were collected for total protein extraction and various analyses.

Western blotting

Equal amounts of total protein (10–30 μg; from the preventative study groups) from each sample was electrophoresed on 10.5% acrylamide gels, as described previously.[13] Western blot analyses were then performed with primary polyclonal antibodies to either pERK1/2 (Thr202/Tyr204; Cell Signaling Technology, Danvers, MA), unphosphorylated nNOS (BD Biosciences, San Jose, CA), pnNOS (Ser1417; Pierce Biotechnology, Rockford, IL), TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA), and the AT_2R (Santa Cruz Biotechnology); primary monoclonal antibodies to pSmad2 (Ser465/467; Cell Signaling Technology), α-SMA (Dako Corporation, Carpinteria, CA), or MMP-13 (Abcam, Cambridge, MA); and the appropriate secondary antibodies. Membranes probed with pERK1/2 and pSmad2 were stripped and reprobed with total ERK1/2 and (unphosphorylated) Smad2, respectively, whereas α-tubulin levels (Millipore Corporation, Bedford, MA) were additionally assessed to demonstrate equivalent loading of samples. Blots detected with the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) were quantified by densitometry with a GS710 Calibrated Imaging Densitometer and Quantity-One software (Bio-Rad Laboratories, Richmond, CA). The density of each parameter was corrected for α-tubulin protein levels (or total ERK1/2 and Smad2 levels for pERK1/2 and pSmad2, respectively) and expressed relative to the untreated control group (*in vitro* studies) or the day 5–injured $AT_2R^{+/+}$ mouse group (*in vivo* studies), defined as 1 in each case.

Gelatin zymography

Conditioned media or tissue extracts from the preventative study groups were incubated with 5 mmol/l amino-phenyl mercuric acetate (Sigma-Aldrich) for 6 h at 37 °C, to stimulate the activation of latent MMPs, before gelatin zymography. Equal volumes of

samples were electrophoresed on gelatin zymographs, as detailed before.[13] Gelatinolytic activity was indicated by clear bands and assessed by densitometry, as described above.

Hydroxyproline assay

Equivalent tissue portions (containing cortex and medulla) from $AT_2R^{+/+}$ and $AT_2R^{-/y}$ mice were lyophilized to dry weight, hydrolyzed in 6 mmol/l hydrochloric acid, and assessed for hydroxyproline content, as described previously.[12,19,37] Hydroxyproline values were then multiplied by a factor of 6.94 to extrapolate total collagen content (as hydroxyproline represents ~14.4% of the amino-acid composition of collagen in most mammalian tissues),[44] which was then divided by the dry weight tissue to yield collagen concentration (% collagen content/dry weight tissue; corrected for the size of the tissue portion analyzed).

Immunohistochemistry

Renal collagen IV and collagen I were identified from paraffin-embedded kidney sections from each of the animals studied, using goat anti-collagen IV (Southern Biotechnology, Birmingham, AL) and rabbit anti-collagen I (Biodesign International, Saco, ME) primary antibodies, respectively.[37] Binding was visualized with the avidin–biotin complex (ABC Elite; Vector, Burlingame, CA) and 3,3′-diaminobenzidine (Sigma-Aldrich), and was morphometrically assessed using point-counting.[37] Using an eye-piece graticule, a minimum of 10 fields at ×20 original magnification were counted, with results being expressed as a percentage of points with positive immunohistochemical staining. Equivalent areas of renal cortex were assessed in each animal, with glomeruli and large vessels excluded from analysis.

BRET assays

BRET experiments were performed in HEK293 cells as described previously.[45–48] For BRET saturation assays,[48] cells were co-transfected with a constant amount of Rluc8-tagged AT_2R receptors and increasing amounts of Venus-tagged RXFP1 receptors. The expression levels of Rluc8- and Venus-tagged constructs for each BRET experiment ($n = 3$ independent experiments) were detected by luminescence (LUMIstar; BMG Labtech, Mornington, VIC, Australia) and fluorescence (Envision; Perkin-Elmer, Waltham, MA) measurements, respectively. The actual Receptor-Venus/Receptor-Rluc8 expression ratios were then plotted.

For real-time kinetic BRET assays,[45–47] cells were assayed before and after treatment with H2 relaxin (30 nmol/l) and/or Ang II (1 μmol/l) or vehicle (phenol red-free + 10% FBS + 0.01% bovine serum albumin) for each Receptor-Venus/Receptor-Rluc8 expression ratio. The ligand-induced BRET signal was calculated by subtracting the ratio of emission through the 'acceptor wavelength window' over emission through the 'donor wavelength window' for a vehicle-treated cell sample from the same ratio for a second aliquot of the same cells treated with agonist, as described previously.[45–47]

Competition-binding assays

Whole-cell competition binding assays were conducted in HEK293 cells stably transfected with the AT_2R.[49–51] The unlabeled ligands used were as follows: CGP42112 (AT_2R agonist), candesartan (AT_1R antagonist), and H2 relaxin at concentrations ranging from 1pmol/l to 1 μmol/l, which were prepared in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin (Dulbecco's modified Eagle's medium + 0.1% bovine serum albumin) (DMEM + 0.1% BSA) on the day of the experiment. For each experiment, each ligand concentration was tested in triplicate. The ability of each ligand to inhibit specific binding of [^{125}I]Sar^{1}Ile^{8}Ang II was assessed. Nonlinear regression of the data was performed using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA).

AlphaScreen pERK1/2 accumulation assays

Parental HEK293 cells and HEK293 cells expressing RXFP1 (HEK-RXFP1) were transiently transfected with AT_2Rs using polyethylenimine at a ratio of 1:6 (DNA:polyethylenimine). Receptor-mediated ERK1/2 phosphorylation (pERK1/2) was determined 48 h after transfection using the ERK1/2 SureFire kit (TGR Biosciences, Hindmarsh, Australia) according to the manufacturer's instructions.[24] Briefly, cells were seeded at 30,000 cells per well and allowed to grow overnight at 37 °C in 5% CO_2. Before stimulation, the cells were serum-starved for 6 h. The cells were stimulated with vehicle (20 mmol/l sodium acetate buffer, pH 5.0) or relaxin (30 nmol/l) in the absence or presence of PD123319 (1 μmol/l) over 60 min to determine the time course of pERK1/2. Cells exposed to PD123319 were pretreated with the AT_2R antagonist for 30 min. Data were normalized to the maximal response elicited by 10% FBS, determined at 5 min.

Statistical analysis

Results were analyzed by one-way analysis of variance followed by the Newman–Kuels *post hoc* test for multiple comparisons between groups, using GraphPad Prism 5.03 (GraphPad Software). All data are expressed as the mean ± standard error of the mean (s.e.m.), with a value of $P < 0.05$ regarded as statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figures S1. Full Western blots of TGF-β1, pSmad2, Smad2, α-SMA and α-tubulin.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Eddy AA. Progression in chronic kidney disease. *Adv Chronic Kidney Dis* 2005; **12**: 353–365.
2. Wynn T. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008; **214**: 199–210.

3. Eddy AA. Molecular insights into renal interstitial fibrosis. *J Am Soc Nephrol* 1996; **7**: 2495–2508.
4. Mezzano SA, Ruiz-Ortega M, Egido J. Angiotensin II and renal fibrosis. *Hypertension* 2001; **38**: 635–638.
5. Stahl PJ, Felsen D. Transforming growth factor-β, basement membrane, and epithelial–mesenchymal transdifferentiation. *Am J Pathol* 2001; **159**: 1187–1192.
6. Bathgate RAD, Hsueh AJ, Sherwood OD. Physiology and molecular biology of the relaxin peptide family. In: Neill JD (eds). *Knobil and Neill's Physiology of Reproduction*, 3rd edn Raven Press: New York, NY, 2006, pp 679–770.
7. Samuel CS, Hewitson TD, Unemori EN *et al.* Drugs of the future: the hormone relaxin. *Cell Mol Life Sci* 2007; **64**: 1539–1557.
8. Samuel CS, Hewitson TD. Relaxin and the progression of kidney disease. *Curr Opin Nephrol Hypertens* 2009; **18**: 9–14.
9. Bennett RG. Relaxin and its role in the development and treatment of fibrosis. *Transl Res* 2009; **154**: 1–6.
10. Teerlink JR, Cotter G, Davison BA *et al.* Serelaxin, recombinant human relaxin-2, for treatment of acute heart failure (RELAX-AHF): a randomised, placebo-controlled trial. *Lancet* 2013; **381**: 29–39.
11. Heeg MH, Koziolek MJ, Vasko R *et al.* The antifibrotic effects of relaxin in human renal fibroblasts are mediated in part by inhibition of the Smad2 pathway. *Kidney Int* 2005; **68**: 96–109.
12. Mookerjee I, Hewitson TD, Halls ML *et al.* Relaxin inhibits renal myofibroblast differentiation via RXFP1, the nitric oxide pathway, and Smad2. *FASEB J* 2009; **23**: 1219–1229.
13. Chow BS, Chew EG, Zhao C *et al.* Relaxin signals through a RXFP1-pERK-nNOS-NO-cGMP-dependent pathway to up-regulate matrix metalloproteinases: the additional involvement of iNOS. *PLoS One* 2012; **7**: e42714.
14. Li JH, Zhu HJ, Huang XR *et al.* Smad7 inhibits fibrotic effect of TGF-beta on renal tubular epithelial cells by blocking Smad2 activation. *J Am Soc Nephrol* 2002; **13**: 1464–1472.
15. Zhang HY, Phan SH. Inhibition of myofibroblast apoptosis by transforming growth factor beta(1). *Am J Respir Cell Mol Biol* 1999; **21**: 658–665.
16. Unemori EN, Amento EP. Relaxin modulates synthesis and secretion of procollagenase and collagen by human dermal fibroblasts. *J Biol Chem* 1990; **265**: 106981–110685.
17. Unemori EN, Pickford LB, Salles AL *et al.* Relaxin induces an extracellular matrix-degrading phenotype in human lung fibroblasts *in vitro* and inhibits lung fibrosis in a murine model *in vivo*. *J Clin Invest* 1996; **98**: 2739–2745.
18. Bennett RG, Kharbanda KK, Tuma DJ. Inhibition of markers of hepatic stellate cell activation by the hormone relaxin. *Biochem Pharmacol* 2003; **66**: 867–874.
19. Samuel CS, Unemori EN, Mookerjee I *et al.* Relaxin modulates cardiac fibroblast proliferation, differentiation, and collagen production and reverses cardiac fibrosis *in vivo*. *Endocrinology* 2004; **145**: 4125–4133.
20. Border WA, Noble NA. Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis. *Hypertension* 1998; **31**: 181–188.
21. Rüster C, Wolf G. Angiotensin II as a morphogenic cytokine stimulating renal fibrogenesis. *J Am Soc Nephrol* 2011; **22**: 1189–1199.
22. Jones ES, Vinh A, McCarthy CA *et al.* AT2 receptors: functional relevance in cardiovascular disease. *Pharm Ther* 2008; **120**: 292–316.
23. Carey RM. Cardiovascular and renal regulation by the angiotensin type 2 receptor. *Hypertension* 2005; **45**: 840–844.
24. Van der Westhuizen ET, Werry TD, Sexton PM *et al.* The relaxin family peptide receptor 3 activates extracellular signal-regulated kinase 1/2 through a protein kinase C-dependent mechanism. *Mol Pharmacol* 2007; **71**: 1618–1629.
25. Halls ML, Bathgate RA, Summers RJ. Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. *Mol Pharmacol* 2006; **70**: 214–226.
26. Siragy HM, Carey RM. The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest* 1997; **100**: 264–269.
27. Matsubara H. Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ Res* 1998; **83**: 1182–1191.
28. Nie W, Yan H, Li S *et al.* Angiotensin-(1–7) enhances angiotensin II induced phosphorylation of ERK1/2 in mouse bone marrow-derived dendritic cells. *Mol Immunol* 2009; **46**: 335–361.
29. Savoia C, Ebrahimian T, He Y *et al.* Angiotensin II/AT2 receptor-induced vasodilation in stroke-prone spontaneously hypertensive rats involves nitric oxide and cGMP-dependent protein kinase. *J Hypertens* 2006; **24**: 2417–2422.
30. Yang J, Chen C, Ren H *et al.* Angiotensin II AT(2) receptor decreases AT(1) receptor expression and function via nitric oxide/cGMP/Sp1 in renal proximal tubule cells from Wistar–Kyoto rats. *J Hypertens* 2012; **30**: 1176–1184.
31. Nakajima M, Hutchinson HG, Fujinaga M *et al.* The angiotensin II type 2 (AT2) receptor antagonizes the growth effects of the AT1 receptor: gain-of-function study using gene transfer. *Proc Natl Acad Sci USA* 1995; **92**: 10663–10667.
32. Sasser JM, Molnar M, Baylis C. Relaxin ameliorates hypertension and increases nitric oxide metabolite excretion in angiotensin II but not N^{ω}-nitro-L-arginine methyl ester hypertensive rats. *Hypertension* 2011; **58**: 197–204.
33. Szabo AJ, Wagner L, Erdely A *et al.* Renal neuronal nitric oxide synthase protein expression as a marker of renal injury. *Kidney Int* 2003; **64**: 1765–1771.
34. Ma J, Nishimura H, Fogo A *et al.* Accelerated fibrosis and collagen deposition develop in the renal interstitium of angiotensin type 2 receptor null mutant mice during ureteral obstruction. *Kidney Int* 1998; **53**: 937–944.
35. Svendsen AM, Zalesko A, Kønig J *et al.* Negative cooperativity in H2 relaxin binding to a dimeric relaxin family peptide receptor 1. *Mol Cell Endocrinol* 2008; **296**: 10–17.
36. Siddiquee K, Hampton J, McAnally D *et al.* The apelin receptor inhibits the angiotensin II type 1 receptor via allosteric trans-inhibition. *Br J Pharmacol* 2013; **168**: 1104–1117.
37. Hewitson TD, Ho WY, Samuel CS. Anti-fibrotic properties of relaxin: in vivo mechanism of action in experimental renal tubulointerstitial fibrosis. *Endocrinology* 2010; **151**: 4938–4948.
38. Diamond JR, Ricardo SD, Klahr S. Mechanisms of interstitial fibrosis in obstructive nephropathy. *Semin Nephrol* 1998; **18**: 594–602.
39. Hein L, Barsh GS, Pratt RE *et al.* Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature* 1995; **377**: 744–777.
40. Rodemann HP, Muller GA. Abnormal growth and clonal proliferation of fibroblasts derived from kidneys with interstitial fibrosis. *Proc Soc Exp Biol Med* 1990; **195**: 57–63.
41. Masterson R, Hewitson TD, Kelynack K *et al.* Relaxin down-regulates renal fibroblast function and promotes matrix remodelling *in vitro*. *Nephrol Dial Transplant* 2004; **19**: 544–552.
42. Samuel CS, Zhao C, Bathgate RA *et al.* Relaxin deficiency in mice is associated with an age-related progression of pulmonary fibrosis. *FASEB J* 2003; **17**: 121–123.
43. Gemici B, Tan R, Ongüt G *et al.* Expressions of inducible nitric oxide synthase and cyclooxygenase-2 in gastric ischemia–reperfusion: role of angiotensin II. *J Surg Res* 2010; **161**: 126–133.
44. Gallop PM, Paz MA. Posttranslational protein modifications, with special attention to collagen and elastin. *Physiol Rev* 1975; **55**: 418–487.
45. Kocan M, See HB, Seeber RM *et al.* Demonstration of improvements to the bioluminescence resonance energy transfer (BRET) technology for the monitoring of G protein-coupled receptors in live cells. *J Biomol Screen* 2008; **13**: 888–898.
46. Kocan M, See HB, Sampaio NG *et al.* Agonist-independent interactions between beta-arrestins and mutant vasopressin type II receptors associated with nephrogenic syndrome of inappropriate antidiuresis. *Mol Endocrinol* 2009; **23**: 559–571.
47. Kocan M, Dalrymple MB, Seeber RM *et al.* Enhanced BRET technology for the monitoring of agonist-induced and agonist-independent interactions between GPCRs and beta-arrestins. *Front Endocrinol (Lausanne)* 2010; **1**: 12.
48. Mercier JF, Salahpour A, Angers S *et al.* Quantitative assessment of beta 1- and beta 2-adrenergic receptor homo- and heterodimerization by bioluminescence resonance energy transfer. *J Biol Chem* 2002; **277**: 44925–44931.
49. D'amore A, Black MJ, Thomas WG. The angiotensin II type 2 receptor causes constitutive growth of cardiomyocytes and does not antagonize angiotensin II type 1 receptor-mediated hypertrophy. *Hypertension* 2005; **46**: 1347–1354.
50. Thomas WG, Motel TJ, Kule CE *et al.* Phosphorylation of the angiotensin II (AT(1A)) receptor carboxyl terminus: a role in receptor endocytosis. *Mol Endocrinol* 1998; **12**: 1513–1524.
51. Bosnyak S, Jones ES, Christopoulos A *et al.* Relative affinity of angiotensin peptides and novel ligands at AT(1) and AT(2) receptors. *Clin Sci* 2011; **121**: 297–303.